Modified Form PTO-1390 (11-98)

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (if known. see 37 CFR 1.5)
AGI-127	10/070466

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/24659	8 September 2000 (08.09.00)	10 September 1999 (10.09.99)

TITLE OF INVENTION: COMPOSITIONS AND METHODS FOR MODIFICATION OF SKIN LIPID CONTENT

APPLICANT(S) FOR DO/EO/US: David A. Brown and Daniel B. Yarosh

Applicant herewith submits to the United Sates Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a.. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
    (see Express Mail Information)

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/070466	PCT/US00/24659	AGI-127

17. ☒ The following fees are submitted:

	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1)-(5)):	
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1,040.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**	
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2) paid to USPTO **$740.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**	
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	

ENTER APPROPRIATE BASIC FEE AMOUNT =				$	710.00
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				$	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	28 - 20 =	8	X **$18.00**	$	144.00
Independent claims	3 - 3 =	0	X **$84.00**	$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$280.00**	$	
TOTAL OF ABOVE CALCULATIONS =				$	854.00
Reduction by 1/2 for filing by small entity, if applicable. A Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				$	427.00
SUBTOTAL =				$	427.00
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$	
TOTAL NATIONAL FEE =				$	427.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$	427.00
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of $ 427.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 11-1158 in the amount of $_____ to cover the above fees.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required by this paper, or credit any overpayment to Deposit Account No. 11-1158. A duplicate copy of this sheet is enclosed

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Maurice M. Klee, Ph.D.
Attorney at Law
1951 Burr Street
Fairfield, Connecticut 06430
(203) 255-1400

Maurice Klee
Maurice M. Klee, Ph.D.
Registration Number. 30,399

March 7, 2002
Date

[222] Attorney Docket No. :AGI-127 PCT

IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

International Appl. No.	:	PCT/US00/24659
International Filing Date	:	8 September 2000 (08.09.00)
Priority Date Claimed	:	10 September 1999 (10.09.99)
Title of Invention	:	COMPOSITIONS AND METHODS FOR MODIFICATION OF SKIN LIPID CONTENT
U.S. Serial No.	:	Not Yet Assigned
Applicant (U.S.)	:	David A. Brown and Daniel B. Yarosh

BOX PCT
COMMISSIONER OF PATENTS AND TRADEMARKS
WASHINGTON, D.C. 20231
ATTENTION: DO/EO/US

VERIFIED STATEMENT (DECLARATION)
CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(c)) - SMALL BUSINESS CONCERN

I hereby declare that I am :

( ) the owner of the small business concern identified below:

(X) an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: Applied Genetics Incorporated Dermatics

ADDRESS OF CONCERN: 205 Buffalo Avenue
Freeport, New York 11520

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern,

including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled

COMPOSITIONS AND METHODS FOR
MODIFICATION OF SKIN LIPID CONTENT

by inventors David A. Brown and Daniel B. Yarosh

described in International Application No. PCT/US00/24659
filed September 8, 2000.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). *NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME: None
ADDRESS: ______
( )INDIVIDUAL ( )SMALL BUS. CONCERN ( )NONPROFIT ORGAN.

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small business entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any

patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: Daniel B. Yarosh
TITLE OF PERSON OTHER THAN OWNER: President
ADDRESS OF PERSON SIGNING: Applied Genetics Incorporated Dermatics
205 Buffalo Avenue
Freeport, New York 11520

SIGNATURE: Daniel Yarosh

DATE: March 5, 2002

[222] Attorney Docket No. :AGI-127 PCT

IN THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

International Appl. No.	:	PCT/US00/24659
International Filing Date	:	8 September 2000 (08.09.00)
Priority Date Claimed	:	10 September 1999 (10.09.99)
Title of Invention	:	COMPOSITIONS AND METHODS FOR MODIFICATION OF SKIN LIPID CONTENT
U.S. Serial No.	:	Not Yet Assigned
Applicant (U.S.)	:	David A. Brown and Daniel B. Yarosh

BOX PCT
COMMISSIONER OF PATENTS AND TRADEMARKS
WASHINGTON, D.C. 20231
ATTENTION: DO/EO/US

PRELIMINARY AMENDMENT

Prior to its initial examination, please amend the above-identified application as follows:

IN THE SPECIFICATION

Please insert the following before the first line of the specification:

CROSS REFERENCE TO RELATED APPLICATIONS

This application is the U.S. national phase under 35 USC §371 of International Application No. PCT/US00/24659, filed September 8, 2000, which was published in English under PCT Article 21(2) on March 15, 2001 as International Publication No. WO 01/17523. This application claims the benefit under 35 USC §119(e) of U.S. Provisional Application No. 60/153,378, filed September 10, 1999, the contents of which in its entirety is hereby incorporated by reference.

IN THE CLAIMS:

Please amend the claims as follows:

1. Amend Claims 1-4, 7, 9, 12, 15-16, 29, and 33 as indicated below.
2. Cancel, without prejudice, Claims 8, 11, and 21-28.

1. (amended) A method for increasing the lipid content of the epidermal keratinocytes of the skin of a mammal in need of such increase comprising topically administering a ursolic acid compound encapsulated in liposomes to said skin in an amount effective to increase the lipid content of said epidermal keratinocytes, said liposomes comprising phospholipid bilayer membranes.

2. (amended) The method of Claim 1 wherein (i) the skin of the mammal is skin that exhibits aging as a result of a reduction in the lipid content of its epidermal layer and (ii) the ursolic acid compound is topically administered to said skin in an amount effective to reduce the effects of aging exhibited by said skin.

3. (amended) The method of Claim 1 wherein (i) the skin of the mammal is skin that exhibits photoaging as a result of a reduction in the lipid content of its epidermal layer and (ii) the ursolic acid compound is topically administered to said skin in an amount effective to reduce the effects of photoaging exhibited by said skin.

4. (amended) The method of Claim 1 wherein (i) the skin of the mammal is skin that has atrophied as a result of a reduction in the lipid content of its epidermal layer and (ii) the ursolic acid compound is topically administered to said skin in an amount effective to reduce the atrophy of said skin.

7. (amended) The method of Claim 1 wherein (i) the skin of the mammal suffers from ichthyosis or ichthyosiform dermatoses and (ii) the ursolic acid compound is topically administered to said skin in an amount effective to reduce said ichthyosis or ichthyosiform dermatoses.

9. (amended) The method of Claim 1 wherein (i) the mammal has the sensation of dry skin as a result of a reduction in the lipid content of the skin's epidermal layer and (ii) the ursolic acid compound is topically administered to the skin of said mammal in an amount effective to reduce said sensation of dry skin.

12. (amended) The method of Claim 1 wherein the ursolic acid compound is administered in a preparation that includes a water soluble preservative.

15. (amended) The method of Claim 1 wherein the content of at least one member of the ceramide lipid family is increased.

16. (amended) The method of Claim 1 wherein the content of free fatty acids is increased.

29. (amended) A composition for topical administration to the skin of a mammal comprising (i) a ursolic acid compound encapsulated in liposomes and (ii) a medium suitable only for topical administration, wherein said liposomes comprise phospholipid bilayer membranes.

33. (amended) A composition for topical administration to the skin of a mammal comprising (i) a ursolic acid compound encapsulated in liposomes, (ii) at least one other therapeutically active topical compound which is not a ursolic acid compound, and (iii) a medium suitable for topical administration, wherein said liposomes comprise phospholipid bilayer membranes.

A copy of original Claims 1-4, 7, 9, 12, 15-16, 29, and 33 annotated to show the changes made by this amendment is attached as Exhibit A.

REMARKS

This is a §371 national phase filing of PCT Patent Application No. PCT/US00/24659.

To expedite the prosecution of this application, applicants have amended their claims as set forth above to more particularly define the inventive aspects of their work. Thus, independent Claim 1 has been amended to: (1) make explicit that the alteration in lipid content produced by the ursolic acid compound is an increase in lipid content, (2) require that the topical administration of the ursolic acid compound takes place with the compound encapsulated in liposomes, and (3) require that the liposomes comprise phospholipid bilayer membranes.

As a result of these changes, Claim 11 has been canceled, the dependency of Claim 12 has been changed from Claim 11 to Claim 1, and the language of Claims 15-16 has been amended to better conform to the amended language of Claim 1. In addition, the "amount effective to..." language of Claims 21-23 and

27-28 has been included in Claims 2-4, 7, and 9, with Claims 21-23 and 27-28 and their dependent Claims 24 and 25 then being canceled. Also, the claims directed to treatment of acne (i.e., Claims 8 and 26) have been canceled. Finally, Claims 29 and 33 have been amended so that like amended Claim 33, they recite that the liposomes comprise phospholipid bilayer membranes.

With these changes, all of applicants' claims include limitations calling for a ursolic acid compound encapsulated in liposomes wherein the liposomes comprise phospholipid bilayer membranes. At page 15, lines 5-11, of their specification, applicants discuss the importance of this combination as follows:

> Liposomal formulations are preferred because ursolic acid is highly insoluble in many solvents, particularly water, and common emulsifiers such as LECINOL S-10 have little effect. In accordance with the invention, this insolubility problem is addressed by taking advantage of the flat, planar structure of ursolic acid to stack it between the lipid tails in the phospholipid bilayer membranes of liposomes. Due to the charged head-group of the phospholipids, liposomes containing ursolic acid are readily soluble in water.

To applicants' knowledge, there is no disclosure or suggestion in the prior art regarding the use of liposomes to topically administer any ursolic acid compound. Likewise, the foregoing advantage of such use, namely, the solution to the solubility problem, is not disclosed or suggested in the art. Accordingly, applicants believe that their claims are both novel and nonobvious in view of the prior art and thus properly patentable.

Consideration of the foregoing in connection with the examination of this application is respectfully requested.

Respectfully submitted,

Date: March 7, 2002 Maurice Klee

Maurice M. Klee, Ph.D.
Reg. No. 30,399
Attorney for Applicant
1951 Burr Street
Fairfield, CT 06430
(203) 255-1400

Exhibit A
Annotated Copy of Claims 1-4, 7, 9, 12, 15-16, 29, and 33

1. (amended) A method for increasing [altering] the lipid content of the epidermal keratinocytes of the skin of a mammal in need of such increase [alteration] comprising topically administering a ursolic acid compound encapsulated in liposomes to said skin in an amount effective to increase [alter] the lipid content of said epidermal keratinocytes, said liposomes comprising phospholipid bilayer membranes. [the epidermis of said skin.]

2. (amended) The method of Claim 1 wherein (i) the skin of the mammal is skin that exhibits aging as a result of a reduction in the lipid content of its epidermal layer and (ii) the ursolic acid compound is topically administered to said skin in an amount effective to reduce the effects of aging exhibited by said skin.

3. (amended) The method of Claim 1 wherein (i) the skin of the mammal is skin that exhibits photoaging as a result of a reduction in the lipid content of its epidermal layer and (ii) the ursolic acid compound is topically administered to said skin in an amount effective to reduce the effects of photoaging exhibited by said skin.

4. (amended) The method of Claim 1 wherein (i) the skin of the mammal is skin that has atrophied as a result of a reduction in the lipid content of its epidermal layer and (ii) the ursolic acid compound is topically administered to said skin in an amount effective to reduce the atrophy of said skin.

7. (amended) The method of Claim 1 wherein (i) the skin of the mammal suffers from ichthyosis or ichthyosiform dermatoses and (ii) the ursolic acid compound is topically administered to said skin in an amount effective to reduce said ichthyosis or ichthyosiform dermatoses.

9. (amended) The method of Claim 1 wherein (i) the mammal has the sensation of dry skin as a result of a reduction in the lipid content of the skin's epidermal layer and (ii) the ursolic acid compound is topically administered to the skin of said mammal in an amount effective to reduce said sensation of dry skin.

12. (amended) The method of Claim 1 [11] wherein the ursolic acid compound is administered in a preparation that includes a water soluble preservative.

15. (amended) The method of Claim 1 wherein the content [in the epidermal layer of the skin] of at least one member of the ceramide lipid family is increased.

16. (amended) The method of Claim 1 wherein the content of free fatty acids [acid content of the epidermal layer of the skin] is increased.

29. (amended) A composition for topical administration to the skin of a mammal comprising (i) a ursolic acid compound encapsulated in liposomes and (ii) a medium suitable only for topical administration, wherein said liposomes comprise phospholipid bilayer membranes.

33. (amended) A composition for topical administration to the skin of a mammal comprising (i) a ursolic acid compound encapsulated in liposomes, (ii) at least one other therapeutically active topical compound which is not a ursolic acid compound, and (iii) a medium suitable for topical administration, wherein said liposomes comprise phospholipid bilayer membranes.

COMPOSITIONS AND METHODS FOR MODIFICATION OF SKIN LIPID CONTENT

BACKGROUND OF THE INVENTION

1. Field of the Invention

As known in the art, the epidermis and dermis of mammalian skin contain different cell types, perform different functions, and have different chemical compositions. A particularly important difference between these layers is their lipid concentrations. The dermis contains fibroblasts which produce collagen and other proteins, but very little lipid. The epidermis, on the other hand, contains keratinocytes which, among other things, produce lipids, but essentially no collagen. The collagen produced by the fibroblasts provides tensile strength to the skin. The lipids produced by the kerotinocytes provide a barrier between the living tissue and the outside world.

The present invention relates to modifying the lipid content of skin for purposes of altering appearance, improving function, improving vitality, reversing the effects of aging, reversing the effects of photodamage, or treating disease by topical administration of ursolic acid, ursolic acid analogs, derivatives of ursolic acid, derivatives of ursolic acid analogs, or combinations thereof. Because the skin lipids are located in the epidermis, this modification of the lipid content of the skin takes place in that layer. For ease of reference, ursolic acid, ursolic acid analogs, derivatives of ursolic acid, derivatives of ursolic acid analogs, or combinations thereof will be referred to herein as simply a "ursolic acid compound". The ursolic acid compound can be encapsulated in liposomes or administered in other formulations suitable for topical administration.

2. Description of Related Art

A. Patents

U.S. Patent 4,857,554, Methods for Treatment of Psoriasis, is directed to treating psoriasis by applying an ointment containing ursolic acid and oleanolic acid dispersed in a petroleum jelly/lanolin carrier.

U.S. Patent 4,530,934, Pharmaceutically Active Ursolic Acid Derivatives, is directed to using active derivatives of ursolic acid to treat ulcers.

U.S. Patent 3,903,089, Ursolic Acid Derivatives, is directed to the synthesis of ursolic acid derivatives and analogs.

U.S. Patent 5,624,909, Derivatives of Triterpenoid Acids as Inhibitors of Cell-adhesion Molecules ELAM-1 (e-selectin) and LECAM-1 (I-selectin), is directed to alleviating inflammation by administration of triterpenoid acid derivatives.

U.S. Patent 5,314,877, Water-soluble Pentacyclic Triterpene Composition and Method for Producing the Same, is directed to making ursolic acid, oleanolic acid, and related triterpenoids soluble in water by formulation in cyclodextrins.

U.S. Reissue Patent RE036068, Methods for Treatment of Sundamaged Human Skin with Retinoids, is directed to reversing the effects of photodamage by topical application of retinoids.

U.S. Patent 5,051,449, Treatment of Cellulite with Retinoids, is directed towards retarding or reversing cellulite accumulation in skin by topical application of retinoids.

U.S. Patent 5,556,844, Pharmaceutical or Cosmetic Composition Containing a Combination of a Retinoid and a Sterol, is directed towards treatment of disorders of epidermial keratinization, epithelial proliferation, or disorders of sebaceous function by topical application of retinoids.

U.S. Patent 5,075,340, Retinoic Acid Glucuronide Preparations for Application to the Skin, is directed towards treatment of acne or wrinkled

skin and prevention of retinoid dermatitis by topical application of retinoic acid glucoronides

U.S. Patent 5,837,224, Method of Inhibiting Photoaging of Skin, is directed to reversing the effects of photodamage by topical application of agents that inhibit UVB-inducible matrix metalloproteinase.

B. Publications

Tokuda, H., H. Ohigashi, K. Koshimizu, and Y. Ito. 1986. Inhibitory effects of ursolic and oleanolic acid on skin tumor promotion by 12-*O*-tetradecanoyhlphorbol-13-acetate. Cancer Lett. 33:279-285.

Ponec, M., and A. Weerheim. 1990. Retinoids and lipid changes in keratinocytes. Meth. Enzymol. 190:30-41.

Griffiths, C. E. M., A. N. Russman, G. Majmudar, R. S. Singer, T. A. Hamilton, and J. J. Voorhees. 1993. Restoration of collagen formation in photodamaged human skin by tretinoin (retinoic acid). New Engl. J. Med. 329:530-5.

Kligman, A. M., and J. J. Leyden. 1993. Treatment of photoaged skin with topical tretinoin. Skin Pharmacol. 6 (Suppl.1):78-82).

Huang, M.-T., C.-T. Ho, Z. Y. Wang, T. Ferraro, Y.-R. Lou, K. Stauber, W. Ma, C. Georgiadis, J. D. Laskin, and A. H. Conney. 1994. Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid. Cancer Res. 54:701-708.

Liu, J. 1995. Pharmacology of oleanolic acid and ursolic acid. J. Ethanopharmacol. 49:57-68.

Manez, S., C., C. Recio, R. M. Giner, and J.-L. Rios. 1997. Effect of selected triterpenoids on chronic dermal inflammation. Eur. J. Pharmacol. 334:103-105.

Ponec, M., A. Weerheim, J. Kempenaar, A. Mulder, G. S. Gooris, J. Bouwstra, and A. M. Mommaas. 1997. The formation of competent barrier lipids in reconstructed human epidermis requires the presence of Vitamin C. J. Invest. Dermatol. 109:348-355.

Griffiths, C. E. M. 1999. Drug treatment of photoaged skin. Drugs & Aging 14:289-301.

Japanese Patent Publication No. 11-5727, published January 12, 1999, describes the use of ursolic acid in combination with retinols in a final cosmetic product to increase dermal collagen. As discussed above, collagen is located and produced in the dermis by fibroblasts. The present invention, on the other hand, is concerned with modifying the content of lipids located and produced in the epidermis by kerotinocytes

3. Epidermal Lipid Composition and Alterations During Differentiation

The epidermis of skin contains a number of lipids that are altered during differentiation as follows (see Downing et al., 1993, p210-221, In: Dermatology in General Medicine):

(i) Phospholipids: Most common are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and sphingomyelin. Phospholipids are the most abundant lipids in the basal layers of the epidermis, but decrease towards the surface of the skin so much so that they are one of the least abundant lipids in the cornified layer. Thus, the phospholipid content of keratinocytes decreases as they differentiate. Conversely, high phospholipid levels are associated with keratinocyte proliferation.

(ii) Free fatty acids: These are primarily saturated and range from 14 to 28 carbons (myristic = 14, palmitic = 16, stearic = 18, arachidic = 20, behenic = 22, lignoceric = 24, cerotic=26). The most common fatty acids in skin are the 22-carbon (15 wt.%) and 24 carbon (27 wt.%) species.

(iii) Triglycerides: These are minor lipid components that serve as intermediates in the transfer of fatty acids from phospholipids to glucosylceramides.

(iv) Glucosylceramides A,B,C-1,C-3,D-1,D-2, C-2: Glucosylceramide A also known as acylglucosylceramide is the

major form, comprising 56 wt.% of this group of lipids. The acyl group in glucosylceramide A is often linoleic acid which is bound to the hydroxyl group of the ω-hydroxyacid (Abraham, Wertz and Downing, 1985, J. Lipid Res. 26:761-766).

(v) Ceramides 1-7: Ceramides are the major lipids in the stratum corneum. They result from deglycosylation of glucosylceramides at the end of the epidermal differentiation process. Ceramide 1 is derived from Glucosylceramide A, Ceramide 2 is derived from Glucosylceramide B, Ceramide 3 is derived from Glucosylceramide C-1, Ceramide 4 is derived from Glucosylceramide C-3, Ceramide 5 is derived from Glucosylceramide D-1, Ceramide 6-A is derived from Glucosylceramide D-2, and Ceramide 6-B is derived from Glucosylceramide C-2. Although Glucosylceramide A comprises 56 wt.% of the glucosyleramides, Ceramide 1 comprises only 8 wt.% of the extractable ceramides because most of it is converted to the ω-hydroxyceramide which permits covalent binding to glutamates of the cornified cell envelope protein, thus, forming a protective barrier around each corneocyte. Ceramide 2 comprises 42 wt.% of this group. Ceramide 6 (phytosphingosine) and ceramide 7 (6-hydroxy-4-sphingenine) together comprise 20 wt.%.

(vi) Cholesterol: This lipid increases as keratinocytes differentiate so that it comprises 30 mol% of stratum corneum lipids (Schaefer and Redelmeier, 1996, Skin Barrier).

(vii) Cholesterol sulfate: This lipid increases cell cohesiveness by forming intercellular cholesterol sulfate calcium bridges.

(viii) Cholesterol esters: During the latter stages of epidermal differentiation, phospholipids are degraded liberating fatty acids which are utilized to produce cholesterol esters.

Examination of lipids in serial sections through pig skin showed the following (Cox and Squier, 1986, J. Invest. Dermatol. 87:741-744): (i) increases of both glucosylceramides and ceramides towards the surface layers, but decreases of glucosylceramides concomitant with increases of ceramides at the outermost layers, (ii) decreases of phospholipids towards the surface layers of the skin (iii) progressive increases of triglycerides, cholesterol and cholesterol esters towards the surface layers of the skin, (iv) progressive increase of cholesterol sulfate and then a sudden decrease at the outermost layer (related to desquamation by a sulfatase).

Ponec and Weerheim (1990, Meth. Enzymol. 190:30-41) reviewed the literature and state that normal epidermal terminal differentiation is marked by depletion of phospholipids, with increase of sterols and certain classes of sphingolipids, with the final stratum corneum lipid products of differentiation consisting mainly of ceramides and nonpolar lipids. Overall, the flow of fatty acids during differentiation appears to be from phospholipids, to triglycerides, to ceramides, and finally hydroxyceramides (Swartzendruber et al., 1987, J. Invest. Dermatol. 88:709-713; Ponec et al., 1997, J. Invest. Dermatol. 109:348-355). Thus the metabolic lipid flow during differentiation appears to be towards formation of hydroxyceramides in the stratum corneum. Hydroxyceramides are linked to involucrin via its numerous glutamate residues (20%) during cornification, resulting in the highly effective barrier function of the skin (Swartzendruber et al., 1987, J. Invest. Dermatol. 88:709-713).

4. <u>Agents Shown to Alter Epidermal Lipids</u>

Retinoic acid is able to reverse the alterations of lipid synthesis that occur during differentiation, resulting in a 3-4-fold increase in phospholipids, a 3-fold decrease in sphingolipids (most notably, ceramides), a 9-fold decrease of acylceramides, a near 2-fold decrease of cholesterol and cholesterol sulfate, a 6-fold decrease of lanosterol, and a 3-fold decrease of FFA in living skin equivalents (Ponec and Weerheim, 1990, Meth. Enzymol. 190:30-41). Thus, it would appear that there are marked differences

between the terminal differentiation that occurs naturally in skin, and the cellular reprogramming that occurs as a result of treatment with retinoic acid.

Vitamin C (50 ug/ml) has been found to result in increases of glucosylceramides and ceramides, most notably ceramides 6 and 7 in living skin equivalents (Ponec et al., 1997, J. Invest. Dermatol. 109:348-355). These increases were accompanied by increased barrier function. Since Vitamin E had no effect on lipid composition even though it is hydrophobic, it was concluded that the main role of Vitamin C is as a donor of hydroxyl groups to sphingoid bases and fatty acids for the formation of protein-bound hydroxyceramides (Ponec et al., 1997, J. Invest. Dermatol. 109:348-355).

5. Effect of Aging on Epidermal Lipids

All major species of epidermal lipids are decreased during the aging process. Particular attention has been paid to the reductions of the ceramide fraction since this results in a notable loss of barrier function with age (reviewed in Rogers et al., 1996, Arch. Dermatol. Res. 288:765-770). However, the percentage ratio of each of the major classes of lipids is unchanged during aging, even though total epidermal lipids are decreased by 30% in the aged (Rogers et al., 1996, Arch. Dermatol. Res. 288:765-770). The most important change of epidermal lipids that occurs with age is related to altered ratios of free fatty acids that result in reductions in ceramide 1 lineolate (Rogers et al., 1996, Arch. Dermatol. Res. 288:765-770). Reductions of ceramide 1 lineolate have been linked to dry skin, atopic dermatitis, and acne (reviewed in Rogers et al., 1996, Arch. Dermatol. Res. 288:765-770).

6. Effect of Photodamage on Epidermal Lipids

Long-term (3 week) daily treatment with either UVA (50 J/cm^2) or UVB (124 mJ/cm^2) has been shown to result in an approximate 2-fold increase of total epidermal lipids in human skin, with increases in the triglyceride, free fatty acid, alkane, squalene, and ceramide fractions (Wefers et al., 1991, J. Invest. Dermatol. 96:959-962). No changes were

found in the sterol, cholesterol, cholesterol ester or cholesterol sulfate fractions (Wefers et al., 1991, J. Invest. Dermatol. 96:959-962). Phospholipids were not examined in this study. In contrast to these results, shortly after exposure (24-48 hr), UVA (50 J/cm^2) resulted in a decrease of the ceramide fraction of living skin and an increase in the relative proportion of phospholipids (Robert et al., 1999, Int. J. Radiat. Biol. 75:317-26). Similarly, shortly after exposure (24-48 hrs), UV-B (0.15 J/cm^2) resulted in a marked depletion of ceramides (Holleran et al., 1997, Photoderm. Photoimmunol. Photomed. 13:117-128). However, unlike UV-A, short-term exposure to UV-B also resulted in a marked (>2-fold) depletion of phospholipids (Holleran et al, 1997, above).

7. Methods to Treat Aged and Photodamaged Skin

Retinoic acid is well known as an agent for treatment of photoaged skin. Topical retinoic acid has been shown to restore collagen I levels that are reduced in photodamaged skin (Griffiths et al., 1993, New Engl. J. Med. 329:530-5). Restoration of collagen I levels correlate with a reduction of fine wrinkles in skin (Griffiths et al., 1993, New Engl. J. Med. 329:530-5). Although retinoids have been shown to alter lipids in cultured skin equivalents (Ponec and Weerheim, 1990, Meth. Enzymol. 190:30-41), there are no reports indicating that retinoids reverse aging or photodamage by altering lipid levels. In part, this may be because retinoids reduce ceramide levels in skin equivalents (Ponec and Weerheim, 1990, Meth. Enzymol. 190:30-41), and reduce the thickness of the stratum corneum when applied topically to human skin (Kligman and Leyden, 1993, Skin Pharmacol. 6,Suppl.1:78-82), which could exacerbate the depletion of ceramides and barrier function that occurs in the aged.

8. Pharmacological Uses of Ursolic Acid

Ursolic acid is pentacyclic triterpene compound known to have a number of pharmacological effects (reviewed in Liu, 1995, J. Ethanopharmacol. 49:57-68). Ursolic acid is closely related to steroids since both are derived from the cyclization of squalene (Suh et al., 1998, Cancer

Res. 58:717-723). It is found in the waxy coating of fruit and in the leaves of many plants, such as heather and rosemary. It is insoluble in most common solvents and as a result it is not widely used. In fact, commercial extraction processes for plant leaves fail to recover measurable levels of ursolic acid.

Ursolic acid has been characterized as an inhibitor of lipoxygenase and cyclooxygenase in inflammatory cells (Najid et al., 1992, FEBS 299:213-217; Suh et al., 1998, Cancer Res. 58:717-723). As such, ursolic acid is expected to have usefulness as an anti-inflammatory agent. Ursolic acid has been shown to inhibit chronic dermal inflammation induced by phorbal esters in an animal model (Manez et al., 1997, Eur. J. Pharmacol. 334:103-105). Ursolic acid has also been shown to inhibit induction of inducible nitric oxide synthase in macrophages (Suh et al., 1998, Cancer Res. 58:717-723), which may contribute to its anti-inflammatory activity.

Ursolic acid has also been shown to induce differentiation and growth arrest of several types of cells, suggesting that it may be useful as a chemotherapeutic differentiation agent (Es-Saady et al., 1996, Cancer Lett. 106:193-197; Hsu et al., 1997, Cancer Lett. 111:7-13; Es-Saady et al., 1996, Anticancer Res. 16:481-486; Paik et al., 1998, Arch. Pharm. Res. 21:398-405). Ursolic acid has also been shown to induce apoptosis in tumor cells (Baek et al., 1997, Int. J. Cancer 73:725-728). Both ursolic acid and oleanolic acid, a closely related structural analog of ursolic acid, have been shown to inhibit tumor promotion induced in mouse skin by phorbal esters (Tokuda et al., 1986, Cancer Lett. 33:279-285; Huang et al., 1994, Cancer Res. 54:701-708). Both compounds have also been shown to prevent lipid peroxidation, which may inhibit free radical damage during cancer initiation and promotion (Balanehru and Nagarajan, 1991, Biochem. Int. 24:981-990).

Ursolic acid also downregulates matrix metalloproteinases (Cha et al., 1998, Oncogene 16:771-778) and elastase (Ying et al., 1991, Biochem. J. 277:521-526) which may provide a mechanism for preventing tumor

invasion (Cha et al., 1996, Cancer Res. 56:2281-84), and, inflammation related damage in skin (Ying et al., 1991, Biochem. J. 277:521-526).

Ursolic acid and a number of triterpenoid derivatives have been shown to have hypolipidemic and anti-atherosclerotic properties (reviewed in Liu, 1995, J. Ethanopharmacol. 49:57-68). Ursolic acid and oleanolic acid lowered blood cholesterol and β-lipoprotein levels 40-50% in animal models of atherosclerosis (reviewed in Liu, 1995, J. Ethanopharmacol. 49:57-68). Consistent with this prior art understanding, topical ursolic acid has been proposed for use in the treatment of psoriasis, a condition characterized by hyperproliferation and inflammation of the epidermis (US patent 4,857,554). In fact, these prior results that ursolic acid and its analogs decrease lipid production and may be used in treatment of the hyperproliferation of psoriasis teach away from the present invention, and make the discovery of the opposite effects unexpected and novel. Contrary to the findings in the literature and the understanding of the prior art, we have discovered that ursolic acid increases the production of lipids, especially ceramides and phospholipids, by keratinocytes of the skin.

SUMMARY OF THE INVENTION

The present invention provides a method for altering the lipid content of mammalian skin by administering an effective amount of a ursolic acid compound to the skin of a mammal (e.g., a human) in need of such a treatment, e.g., to skin which is aged, photoaged, atrophied, etc. As discussed above, because the lipids of the skin are located in the epidermal layer, the alteration of the lipid content of the skin takes place in that layer.

In another aspect, the present invention provides a method for reversing certain aspects of the photoaging or aging process in mammalian skin and, in yet another aspect, the present invention provides a method for improving function, increasing barrier function, improving vitality, or treating lipid deficient diseases of mammalian skin, which comprises topical application of:

(a) an effective amount of a ursolic acid compound in a suitable medium for topical administration, e.g., a lotion, gel, or the like;

(b) an effective amount of a ursolic acid compound encapsulated in liposomes; and/or

(c) an effective amount of a ursolic acid compound encapsulated in liposomes and incorporated into a suitable medium for topical administration, e.g., a lotion, gel, or the like.

As discussed below, because ursolic acid compounds are highly insoluble in many solvents, including water, administration of such compounds in liposomes is a particularly preferred embodiment of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows micrographs of cultured normal human epidermal keratinocytes (NHEK) that were: (a) Untreated, (b) treated with 1% (volume/volume) Empty Liposomes, (c & d) treated with 1% 4 mM Ursolic Acid (URA) Liposomes, which are identical to Empty Liposomes except for incorporation of ursolic acid at a concentration of 4 mM - the final concentration of ursolic acid in media is 40 uM. Details are described in Example 1.

Figure 2 shows the amount of phosphatidylcholine per cell relative to Untreated cultured normal human epidermal keratinocytes (NHEK), cultured NHEK treated with 1% (volume/volume) Empty Liposomes, or cultured NHEK treated with 1% 4 mM Ursolic Acid Liposomes. Details are described in Example 1.

Figure 3 shows the amount of phosphatidylethanolamine per cell relative to Untreated cultured normal human epidermal keratinocytes (NHEK), cultured NHEK treated with 1% (volume/volume) Empty Liposomes, or cultured NHEK treated with 1% 4 mM Ursolic Acid Liposomes. Details are described in Example 1.

Figure 4 shows the amount of free fatty acid per cell relative to Untreated cultured normal human epidermal keratinocytes (NHEK), cultured NHEK treated with 1% (volume/volume) Empty Liposomes, or cultured NHEK treated with 1% 4 mM Ursolic Acid Liposomes. Details are described in Example 1.

Figure 5 shows the amount of total ceramides per cell relative to Untreated cultured normal human epidermal keratinocytes (NHEK), cultured NHEK treated with 1% (volume/volume) Empty Liposomes, or cultured NHEK treated with 1% 4 mM Ursolic Acid Liposomes. Details are described in Example 1

Figure 6 shows the amount of total glycosylceramides per cell relative to Untreated cultured normal human epidermal keratinocytes (NHEK), cultured NHEK treated with 1% (volume/volume) Empty Liposomes, or cultured NHEK treated with 1% 4 mM Ursolic Acid Liposomes. Details are described in Example 1.

Figure 7 shows the amount of cholesterol per cell relative to Untreated cultured normal human epidermal keratinocytes (NHEK), cultured NHEK treated with 1% (volume/volume) Empty Liposomes, or cultured NHEK treated with 1% 4 mM Ursolic Acid Liposomes. Details are described in Example 1.

Figure 8 shows the amount of extractable ceramides in human skin following treatment with 0.3% or 1% Ursolic Acid Liposomes in a hydrogel Lotion for 3 and 11 days, relative to adjacent areas treated with Empty Liposome Lotion. These concentrations of Ursolic Acid Liposomes resulted in a final concentration of ursolic acid in the hydrogel Lotion of 10μM and 30μM, respectively.

DETAILED DESCRIPTION OF THE INVENTION

The compounds and compositions of the present invention effectively and efficiently increase the phospholipid content of normal human epidermal keratinocytes. Furthermore, the compounds and compositions of the present invention increase the free fatty acid content and the ceramide

and glycosylceramide content of normal human epidermal keratinocytes. Increases of phospholipid, free fatty acid, ceramide and glycosylceramide content are due to the effects of ursolic acid and are over and above any effects of the lipid carrier.

Increasing the lipid content of epidermal keratinocytes has several consequences. First, increasing the phospholipid content prevents cell senescence and stimulates proliferation, which is manifested as increased cellular viability and increased epidermal thickening. Second, increasing the free fatty acid content relates to increases of several lipid fractions including phospholipids, triglycerides, glucoceramides, ceramides and acylceramides. Increasing the lipid content of epidermal keratinocytes reverses the lipid reductions associated with aging. Third, increasing the glucoceramide, ceramide and acylceramide content of keratinocytes results in an improved barrier function. Improved barrier function, in turn, reduces atopic dermatitis and protects the skin and body from the effects of many agents including ultraviolet irradiation, toxic chemicals, toxins, and irritants. Fourth, preventing cell senescence and increasing barrier function reverses the effects of aging. Fifth, increases of phospholipids directly reverse the depleting effect of UV-B on phospholipids in skin, resulting in increased viability as manifested by cellular proliferation, and reducing the effects of photoaging.

The users of this invention who benefit from its discovery include, among others, those suffering from ichthyosis and ichthyosiform dermatoses, those with acute dry skin, those with skin atrophy and retinoid dermatoses, those with acne and those with aging and photoaging skin. These conditions all have in common a reduction of skin lipids, particularly those produced by keratinocytes and especially ceramides, a reduction in the thickness and uniformity of intact stratum corneum and a loss of skin barrier function. As a consequence, the users of this invention share the pathological conditions of increased transepidermal water loss and a sensation of dry skin.

The active compound according to the present invention is a ursolic acid compound. Examples of such compounds are set forth in Table 1, it being understood that the invention is not limited to the examples of this table but includes all ursolic acid compounds which achieve the beneficial, lipid altering effects of the invention when applied to mammalian skin.

The table shows the chemical structure common to this family of compounds. The reference compound, ursolic acid, contains the substituents at the indicated sites as shown in the second line of the table. Each of the analogs/derivatives listed below ursolic acid differs from the ursolic acid structure only where indicated. Blank cells indicate that the substituents at those sites are identical to ursolic acid. As can be appreciated from the diagram, these compounds share great similarity in the A, B, and C rings of the pentacyclic structure. An addition at sites on any of these rings, such as the hemisuccinate at the R_2 position of ring A in carbenoxolone, reduces the potency of the compound despite the increase in solubility that may be achieved by the addition. On the other hand, many modifications of the E ring, including larger alkyl groups at the R_6 position and substitution of a pentyl for a hexyl ring, can be tolerated. Oxo additions at the R_4 position increase the mineralocorticoid activity of the compound and are to be preferred only when anti-diuresis and water retention is desirable or not harmful.

As discussed above, the methods and compositions of the present invention employ a ursolic acid compound (which, as defined above, can be a combination (mixture) of compounds) as an active ingredient for various uses. In a preferred embodiment, the active ingredient is given topically in an acceptable formulation. A particularly preferred formulation is the incorporation of the ursolic acid compound into liposomes. A variety of different types of lipids at various concentrations can be used to form the liposomes, examples of which can be found in Liposome Technology, ed. Gregory Gregoriadis, CRC Press Inc., Boca Raton, Florida 1984. The present invention also relates to the incorporation of the ursolic acid

compound, either alone or incorporated in liposomes, into lotions, gels, creams or other acceptable formulations conducive to uptake of active ingredients into the epidermis.

Liposomal formulations are preferred because ursolic acid is highly insoluble in many solvents, particularly water, and common emulsifiers such as LECINOL S-10 have little effect. In accordance with the invention, this insolubility problem is addressed by taking advantage of the flat, planar structure of ursolic acid to stack it between the lipid tails in the phospholipid bilayer membranes of liposomes. Due to the charged head-group of the phospholipids, liposomes containing ursolic acid are readily soluble in water.

However, only a limited number of sites within the tails of the lipid bilayer of the liposome membrane are available for stacking ursolic acid. The preferred ratio of ursolic acid to lipid components must be determined by experimentation. For example, the preferred ratio of ursolic acid to phosphatidylcholine and cholesterol is approximately 1.5 (range 1.0 to 3.0):10:1.9 (w/v). At lower concentrations of ursolic acid, the effects on lipid production by the epidermal layer of mammalian skin are not easily evoked. At the higher ranges of ursolic acid concentration, other compounds that partition into the liposome membrane cannot be included in the preparation, because they displace the ursolic acid and lead to its precipitation.

For example, inclusion of the lipophilic preservative phenoxyethanol at the recommended concentration of 1% leads to a ursolic acid precipitate forming in the preparation. Therefore it is difficult to achieve an effective concentration of ursolic acid in liposomes and an effective concentration of a lipophilic preservative. The preferred preservatives that are compatible with ursolic acid liposomes are water-soluble preservatives that do not partition into the liposome membrane. One such water-soluble preservative that can be used in the practice of the invention is potassium sorbate. Other water soluble preservatives can be found in: Cosmetic and

Drug Preservation. Principles and Practices. Ed. J.J. Kabara. Marcel Dekker, Inc. New York, 1984.

Similarly, other additives to a liposomal ursolic acid composition should not displace the ursolic acid from the liposome, and the preferred form of such additives should be water soluble or otherwise sequestered from the liposomes.

The dose regimen will depend on a number of factors which may be readily determined, such as severity and responsiveness of the condition to be treated, but will normally be one or more treatments per day, with treatment lasting from several days to several months, or until the desired response is obtained, or a cure is effected, or a remediation of a condition or a diminution of a disease state is achieved. One of ordinary skill may readily determine optimum dosages, dosing methodologies and repetition rates. In general, the unit dosage for compositions according to the present invention will contain from 0.1 mM to 10 mM of the ursolic acid compound in liposomes or an alternative carrier, with said liposomes or carrier comprising from 1 wt.% to 90 wt.% of a lotion or alternative topical formulation. In some instances, it may be preferable to apply said liposomes or alternative formulations full strength to skin.

If desired, the ursolic acid compound can be combined with other active ingredients in the formulation. For example, it is known that retinoids and topical steroids have the undesirable side effect of skin atrophy. This side effect can be ameliorated by co-administration of a ursolic acid compound with these agents. The co-administration can be performed using a single vehicle, e.g., a lotion, containing both active ingredients or by means of separate vehicles, e.g., separate lotions, which can be administered either simultaneously or sequentially, with either agent being administered first. As another example, a ursolic acid compound can be combined with a sunblock to form a sunscreen product.

The uses of and useful and novel features of the present methods and compositions will be further understood in view of the following non-limiting examples.

Example 1

Preparation of Liposomes

Ursolic acid-containing liposomes and empty liposomes containing no ursolic acid were prepared as follows: 0.393 g phosphatidylcholine (DOOSAN) and 0.077 g cholesterol (AVANTI) were dissolved in 20 ml ethanol, and split into two 10 ml aliquots. Sixty mg ursolic acid (SIGMA-ALDRICH) was then dissolved in one aliquot which was then used to make Ursolic Acid Liposomes. No additions were made to the aliquot designated as Empty Liposomes. Seven ml of each mixture were injected through a 30½G needle into 10 ml cold 1XPBS. The resulting mixture was dialyzed for 2 hr in 2 liters 1XPBS, and then overnight in a fresh batch of 1XPBS. Liposomes were collected and ursolic acid content was measured by high performance thin layer chromatography (HPTLC) using RP-18 $F_{254}S$ HPTLC plates (MERCK). Ursolic acid was dissolved in methanol to make a series of standards. Liposomes were applied directly to HPTLC plates. The mobile phase was 100% methanol. Chromatograms were visualized by spraying with antimony trichloride followed by heating at 100°C for 5 min. Stained chromatograms were photographed and spots were analyzed by QUANTISCAN image analysis software. Ursolic Acid Liposomes contained 4 mM ursolic acid.

Cell Culture and Treatments

Normal human epidermal keratinocytes (NHEK) were obtained from CLONETICS BIOWHITTAKER and cultured in KGM-2 media (CLONETICS-BIOWHITTAKER) according to the manufacturer's instructions. Four days before beginning treatments, 10^5 NHEK were plated in each of six 10 cm^2 CORNING cell culture dishes in 10 ml KGM-2 media. Two days later and on the first day of treatments, media was replaced with 10 ml fresh media.

On the first day of treatments, 100 ul Empty Liposomes were added to each of two dishes, and 100 ul 4 mM Ursolic Acid Liposomes were added to each of two dishes. Thus, the Empty Liposome and 4 mM Ursolic Acid Liposome treatments each received 1% liposomes, and the final concentration of ursolic acid in the Ursolic Acid Liposome treatment was 40 uM. All treatments received fresh media and liposomes on the fourth day following the initiation of treatments.

Cells were harvested 8 days following the initiation of treatments. Media was removed and cells were washed once with 10 ml CLONETICS-BIOWHITTAKER Hank's Buffered Saline Solution (HBSS), then 5 ml HBSS was added, and cells were photographed using a NIKON microscope equipped with camera linked to a NORTHERN EXPOSURE computer imaging system. Following photograph, HBSS was removed, 6 ml CLONETICS Trypsin/EDTA was added, and cells were incubated at 37°C for 6 min until they detached from dishes. Then, 6 ml CLONETICS Trypsin Neutralization Solution was added, and the cells were mixed thoroughly and transferred to a 15 ml conical tube. One-half ml of suspended cells were added to 19.5 ml Isoton II and counted on a model ZBI COULTER counter. The remainder of cells were then pelleted by centrifugation at 178 X g for 5 min. Supernatant was removed and cells were then resuspended in 5 ml 1XPBS, transferred to a PYREX test tube with teflon-lined lid, and pelleted for lipid extraction.

Lipid Extraction Method

Lipids were extracted using procedures developed by Ponec and Weerheim (1990, Meth. Enzymol. 190:30-41), that were a modification of procedures developed by Bligh and Dyer (1959, Canad. J. Biochem. Physiol. 37:911-917). Pelleted cells were extracted by mixing with 2 ml chlorform:methanol (2:1) for 60 min at room temperature (RT) on a LABQUAKE rotary mixer. Cellular debris was pelleted, the supernatant collected, and the pellet re-extracted with 2 ml chloroform:methanol:deionized water (1:2:0.5) for 60 min at 37°C, followed

by 2 ml chloroform:methanol (1:2) for 30 min at RT, 2 ml chloroform:methanol (2:1) for 30 min at RT, and 2 ml chloroform at for 15 min at RT. The combined extracts were mixed with 200 ul 2.5% KCl by vortexing, and then mixed with 2 ml deionized water for 10 min at RT. Both the upper aqueous layer and the bottom extract layer were transferred to clean tubes, and the aqueous layer was re-extracted with 4 ml chloroform by mixing for 10 min at RT. This chloroform extract was combined with the previous extracts. The combined extracts were placed in a 50°C water bath and dried under a stream of nitrogen. The pellet was dissolved in 500 ul chloroform(2):methanol(1) and stored in a teflon lined vial under nitrogen at -20°C.

For analysis of lipids in human skin, lipids were extracted from subjects using a modification of the protocol described by Bontè, F., A. Saunois, P. Pinguet, and A. Meybeck, in Arch. Dermatol. Res. 289:78-82, 1997. Following treatments, the area on the forearm to be extracted was first rinsed with tap water, dried thoroughly and then tape-stripped once with SCOTCH™ 810 MAGIC™ tape. The excised top 1-inch of 50 ml polypropylene conical tubes were used as reservoirs for the solvents during extraction of subjects. The reservoirs were placed and held firmly on the arm and 1 ml of cyclohexane:ethanol (4:1) was added and stirred gently for one minute. Solvent was then removed and placed into a pyrex tube with a teflon-lined lid. One ml of cyclohexane:ethanol (1:1) was then added to the reservoir, stirred for one minute, and then removed and added to the tube containing the first extract. The tubes were then dried at 50°C under nitrogen gas as described above. The dried extracts were dissolved in 200 µl of chloroform:methanol (2:1). The lipid solution was then placed into a small storage tube with a teflon-lined lid, purged with nitrogen gas and stored at –20°C.

Thin Layer Chromatography Methods

Standard solutions were prepared that contained 5 ug/ul each of phosphatidylcholine (PC), phosphatidylethanolamine (PE), cholesterol

(CH), total ceramides (α-hydroxy ceramides + non-hydroxy ceramides)(Cer), total glycosylceramides (GlyCer), and oleic acid, a commonly used reference material for free fatty acids (FFA). These were then serially diluted to 2.5 ug/ul, 1.25 ug/ul, 0.625 ug/ul, 0.313 ug/ul, 156 ng/ul, and 78 ng/ul, and 5 ul of each were run on 250 um thick Silica TLC plates with polyester support (SIGMA-ALDRICH) for PC and PE, or, on 150 um thick Silica HPTLC plates with glass support (SIGMA-ALDRICH) for FFA, CH, Cer, and GlyCer. TLC plates were developed by running chloroform:methanol:deionized water (65:30:5) 60 mm as the mobile phase. HPTLC plates were developed using the following sequence of mobile phases for FFA and CH: (i) chloroform run 15 mm, (ii) chloroform-acetone-methanol (76:8:16) run 10 mm, (iii) chloroform-hexyl acetate-acetone-methanol (86:1:10:4) run 70 mm, (iv) chloroform-acetone-methanol (76:4:20) run 20 mm, (v) chloroform-diethyl ether-hexyl acetate-ethyl acetate-acetone-methanol (72:4:1:4:16:4) run 75 mm, and, (vi) hexane-diethyl ether-ethyl acetate (80:16:4) run 90 mm (Ponec and Weerheim, 1990, Meth. Enzymol. 190:30-41), or, the following sequence of mobile phases for Cer and GlyCer: (i) chloroform-methanol-water (40:10:1) run 30 mm, (ii) chloroform-methanol-glacial acetic acid (190:9:1) run 75 mm, (iii) hexane-diethyl ether-glacial acetic acid (80:20:10) run 75 mm, and (iv) petroleum ether run 85 mm (Kennedy et al., 1996, Pharmaceut. Res. 13:1162-1167). TLC plates were dried, stained with iodine, photographed, and analyzed using a QUANTASCAN computer imaging system. HPTLC plates were dried, sprayed with phosphomolybdic acid, baked at 120°C for 2 min for FFA and CH, or, sprayed with 10% copper sulfate in 8% phosphoric acid for Cer and GlyCer, and analyzed similar to TLC plates. Intensity of standard staining was found to be linear up to at least 0.625 ug/ul, and this was therefore used as a standard on chromatographic runs with samples.

Samples were run using either a standard amount of extract (5 ul), followed by normalization of results to cell number, or by loading an amount of extract equivalent to a standard number of cells (2.5 X 10^4 cells).

Both techniques produced similar results. Results obtained using extracts from a standard number of cells are shown in this example. TLC's and HPTLC's were run, stained and quantified in a fashion identical to that described above for standards. Statistical analysis was done by Student-Newman-Keuls ANOVA using the INSTAT software.

Results and Pharmacological Applications

Visual observation and photographic record (Figure 1) showed NHEK cells treated with 1% 4 mM Ursolic Acid Liposomes were highly vacuolated relative to Untreated cells or cells treated with Empty Liposomes. Vacuolation can result from several conditions including accumulation of lipid (Robert et al., 1999, Int. J. Radiat. Biol. 75:317-326). In light of the lipid analysis below, it is believed that the vacuoles observed here resulted from lipid accumulation. It should be noted that there was little or no increase in vacuolation of NHEK treated with Empty Liposomes, despite the fact that liposomes contain phosphatidlycholine and cholesterol. Thus, the vacuolation observed here is specific for Ursolic Acid Liposome treated cells, indicating that ursolic acid is responsible for induction of vacuolation. Although the photographs in Figure 1 were taken after 8 days of treatment, visual observation indicated that significant vacuolation of Ursolic Acid Liposome treated cells was apparent as soon as 2 days after the initiation of treatments.

Analysis of lipid content by TLC and HPTLC indicated that Ursolic Acid Liposomes resulted in a statistically significant 2-fold increase of phosphatidylcholine (PC) in NHEK relative to Untreated NHEK (Figure 2). There was no increase of PC in NHEK treated with Empty Liposomes despite the fact that these liposomes contain PC. There was a statistically significant 1.3-fold increase of phosphatidylethanolamine (PE) in NHEK treated with Ursolic Acid Liposomes (Figure 3). Treatment of NHEK with Empty Liposomes resulted in a 13% decrease of PE, although this was not statistically significant. Empty liposomes resulted in a 1.15-fold increase of free fatty acids (FFA) while Ursolic Acid Liposomes resulted in 1.3-fold

increases of FFA; only the increase of FFA induced by Ursolic Acid Liposomes was statistically significant (Figure 4). Ursolic acid liposomes dramatically increased total ceramides and glycosylceramides compared to untreated cells (Figures 5 and 6). Neither Empty Liposomes nor Ursolic Acid Liposomes increased cholesterol levels in cells (Figure 7). These findings in keratinocytes are unexpected in view of the reports in the prior art that ursolic acid reduces cholesterol levels and is hypolipidemic.

Treatment of human subjects with lotion containing 10 μM or 30μM liposomal ursolic acid resulted in induction of ceramides, with increases of hydroxy-ceramides generally greater than non-hydroxy-ceramides (Figure 8). In fact, following 3 days of treatment, non-hydroxy ceramides were decreased approximately 12% by 10 μM or 30μM liposomal ursolic acid. In contrast, hydroxy ceramides were increased by approximately 18% following 3 days of treatment with either 10 μM or 30μM liposomal ursolic acid. Following 11 days of treatment, both non-hydroxy and hydroxy ceramides were increased approximately 30% by 10μM liposomal ursolic acid. However, at this same time period, non-hydroxy and hydroxy ceramides were increased only 7% and 18% respectively, by 30 μM ursolic acid. Thus, whereas 30μM liposomal ursolic acid was a more optimal concentration for induction of ceramides in cultured NHEK, 10μM liposomal ursolic acid was a more optimal concentration for induction of ceramides in human skin. However, it should be noted that whereas cell culture treatments were done only once every third day, treatment of human skin was done twice daily. Thus, the ability of liposomal ursolic acid to induce ceramides in human skin may have been saturated by this treatment regimen.

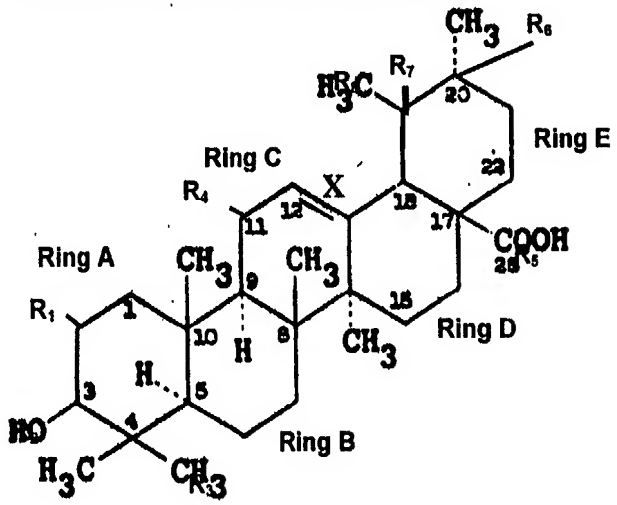
Previous studies have shown that alterations of specific lipids can be associated with functional and aesthetic changes in skin. For example, treatment of skin with retinoids results in increases of phospholipids, a reduction of ceramides, and reduced senescence of epidermal cells. Thus, the viability and vitality of skin is increased while the barrier function is

reduced. Furthermore, it is known that lipid accumulation in epidermal cells is associated with a restoration of a youthful appearance.

Similar to retinoids, the compounds of the present invention result in increased levels of phospholipids in cells. However, incorporation of the compounds of the present invention into liposomes results in accumulation of additional lipids, including total ceramides and glycosylceramides which are extremely important for barrier function. Thus, unlike results with retinoids wherein ceramide levels are decreased (Ponec and Weerheim, 1990, Meth. Enzymol. 190:31-40), the barrier function of skin will not be compromised by the compositions of the present invention. In addition, the compositions will stimulate phospholipid synthesis and thereby increase cell viability. Thus, the topical administration of a ursolic acid compound in accordance with the invention will serve to counteract the effects of aging and photoaging and to treat diseases of dry skin related to impaired barrier function and dysfunctional stratum corneum.

TABLE 1

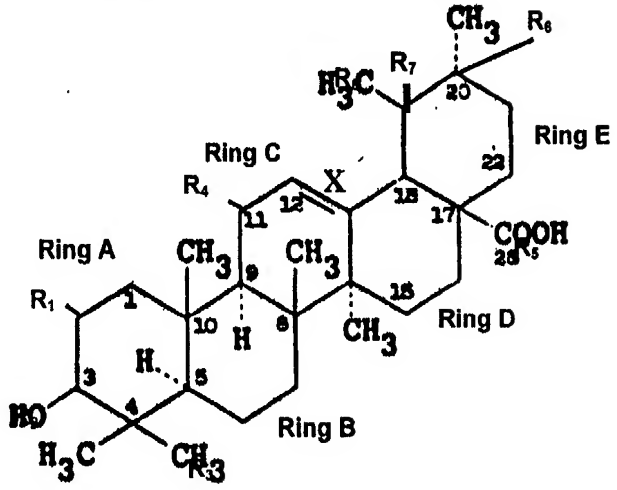
Representative Ursolic Acid Compounds



Name	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	X	Ring E
Ursolic acid	**H**	**OH**	**CH_3**	**H**	**COOH**	**H**	**H**	**CH_3**	**-ene**	**hexyl**
Oleanolic acid						CH_3		H		
Erythrodiol					CH_2OH	CH_3		H		
12,13-epoxyoleanolic acid						CH_3		H	-epoxy	
Uvaol					CH_2OH					
Hederagenin			CH_2OH			CH_3				
Corosolic acid	OH									
Euscapic acid	OH						OH			
18β-glycyrrhetic acid				O=	CH_3	COOH		H		

TABLE 1 (continued)

Representative Ursolic Acid Compounds



Name	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	X	Ring E
Carbenoxolone		$CO(CH_2)_2$-CO_2H		O=		COOH		H		
Glycyrrhetol				O=	CH_3	CH_2OH				
Betulin					CH_2OH		COOH	H		Pentyl
Betulinic acid							COOH	H		Pentyl
Lupane					CH_3		$COCH_2OH$	H		Pentyl
New Lupane					CH_2OH		COCHO	H		Pentyl

Ursolic acid is the reference compound. Blank cells indicate that the site is identical to ursolic acid.

What is claimed is:

1. A method for altering the lipid content of the skin of a mammal in need of such alteration comprising topically administering a ursolic acid compound to said skin in an amount effective to alter the lipid content of the epidermis of said skin.

2. The method of Claim 1 wherein the skin of the mammal is skin that exhibits aging as a result of a reduction in the lipid content of its epidermal layer.

3. The method of Claim 1 wherein the skin of the mammal is skin that exhibits photoaging as a result of a reduction in the lipid content of its epidermal layer.

4. The method of Claim 1 wherein the skin of the mammal is skin that has atrophied as a result of a reduction in the lipid content of its epidermal layer.

5. The method of Claim 4 wherein the reduction in the lipid content of the epidermal layer is a result of the application of at least one retinoid to the skin.

6. The method of Claim 4 wherein the reduction in the lipid content of the epidermal layer is a result of the application of at least one steroid to the skin.

7. The method of Claim 1 wherein the skin of the mammal suffers from ichthyosis or ichthyosiform dermatoses.

8. The method of Claim 1 wherein the skin of the mammal suffers from acne.

9. The method of Claim 1 wherein the mammal has the sensation of dry skin as a result of a reduction in the lipid content of the skin's epidermal layer.

10. The method of Claim 1 wherein the ursolic acid compound is administered in a formulation and the concentration of the ursolic acid compound in that formulation is equal to or less than 30 micromolar.

11. The method of Claim 1 wherein the ursolic acid compound is encapsulated in liposomes.

12. The method of Claim 11 wherein the ursolic acid compound is administered in a preparation that includes a water soluble preservative.

13. The method of Claim 12 wherein the water soluble preservative is potassium sorbate.

14. The method of Claim 1 wherein the ursolic acid compound is ursolic acid.

15. The method of Claim 1 wherein the content in the epidermal layer of the skin of at least one member of the ceramide lipid family is increased.

16. The method of Claim 1 wherein the free fatty acid content of the epidermal layer of the skin is increased.

17. The method of Claim 1 wherein the ursolic acid compound is administered in combination with at least one other active compound which is not a ursolic acid compound.

18. The method of Claim 17 wherein the at least one other active compound is a retinoid.

19. The method of Claim 17 wherein the at least one other active compound is a steroid.

20. The method of Claim 1 wherein the mammal is a human.

21. A method for treating skin of a mammal which exhibits aging as a result of a reduction in the lipid content of its epidermal layer comprising topically administering a ursolic acid compound to said skin in an amount effective to reduce the effects of aging exhibited by said skin.

22. A method for treating skin of a mammal which exhibits photoaging as a result of a reduction in the lipid content of its epidermal layer comprising topically administering a ursolic acid compound to said skin in an amount effective to reduce the effects of photoaging exhibited by said skin.

23. A method for treating skin of a mammal which is atrophied as a result of a reduction in the lipid content of its epidermal layer comprising topically administering a ursolic acid compound to said skin in an amount effective to reduce the atrophy of said skin.

24. The method of Claim 23 wherein the atrophied mammalian skin is a result of treatment with at least one retinoid.

25. The method of Claim 23 wherein the atrophied mammalian skin is a result of treatment with at least one steroid.

26. A method for treating skin of a mammal suffering from acne comprising topically administering a ursolic acid compound to said skin in an amount effective to reduce said acne.

27. A method for treating skin of a mammal suffering from ichthyosis or ichthyosiform dermatoses comprising topically administering a ursolic acid compound to said skin in an amount effective to reduce said ichthyosis or ichthyosiform dermatoses.

28. A method of treating a mammal suffering from the sensation of dry skin as a result of a reduction in the lipid content of its epidermal layer comprising topically administering a ursolic acid compound to skin of said mammal in an amount effective to reduce said sensation of dry skin.

29. A composition for topical administration to the skin of a mammal comprising (i) a ursolic acid compound encapsulated in liposomes and (ii) a medium suitable only for topical administration.

30. The composition of Claim 29 further comprising a water soluble preservative.

31. The composition of Claim 30 wherein the water soluble preservative is potassium sorbate.

32. The composition of Claim 29 wherein the concentration of the ursolic acid compound in the composition is equal to or less than 30 micromolar.

33. A composition for topical administration to the skin of a mammal comprising (i) a ursolic acid compound encapsulated in liposomes,

(ii) at least one other therapeutically active topical compound which is not a ursolic acid compound, and (iii) a medium suitable for topical administration.

34. The composition of Claim 33 wherein the at least one other therapeutically active topical compound is a retinoid.

35. The composition of Claim 33 wherein the at least one other therapeutically active topical compound is a steroid.

36. The composition of Claim 33 further comprising a water soluble preservative.

37. The composition of Claim 36 wherein the water soluble preservative is potassium sorbate.

38. The composition of Claim 33 wherein the concentration of the ursolic acid compound in the composition is equal to or less than 30 micromolar.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 March 2001 (15.03.2001)

PCT

(10) International Publication Number
WO 01/17523 A1

(51) International Patent Classification[7]: A61K 31/19

(21) International Application Number: PCT/US00/24659

(22) International Filing Date:
8 September 2000 (08.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/153,378 10 September 1999 (10.09.1999) US

(71) Applicant *(for all designated States except US)*: **APPLIED GENETICS INCORPORATED DERMATICS** [US/US]; 205 Buffalo Avenue, Freeport, NY 11520 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **BROWN, David, A.** [US/US]; 44 Central Parkway, Merrick, NY 11566 (US). **YAROSH, Daniel, B.** [US/US]; 2915 Shore Drive, Merrick, NY 11566 (US).

(74) Agent: **KLEE, Maurice, M.**; 1951 Burr Street, Fairfield, CT 06430 (US).

(81) Designated States *(national)*: AU, CA, CN, IL, JP, KR, US.

(84) Designated States *(regional)*: European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: COMPOSITIONS AND METHODS FOR MODIFICATION OF SKIN LIPID CONTENT

(57) Abstract: The topical use of ursolic acid compounds to alter the lipid content of mammalian skin is disclosed. The compounds can be encapsulated in liposomes and administered in this form to the skin in, for example, a lotion or a gel. The compounds are effective in, among other things, reducing the effects of aging, photoaging, and skin atrophy, including skin atrophy resulting from the topical use of retinoids and/or steroids. Compositions comprising a ursolic acid compound in combination with another therapeutically active topical compounds, such as, a retinoid or a steroid, are also disclosed.

FIG. 1A

Untreated

FIG. 1B

1% Empty Liposomes

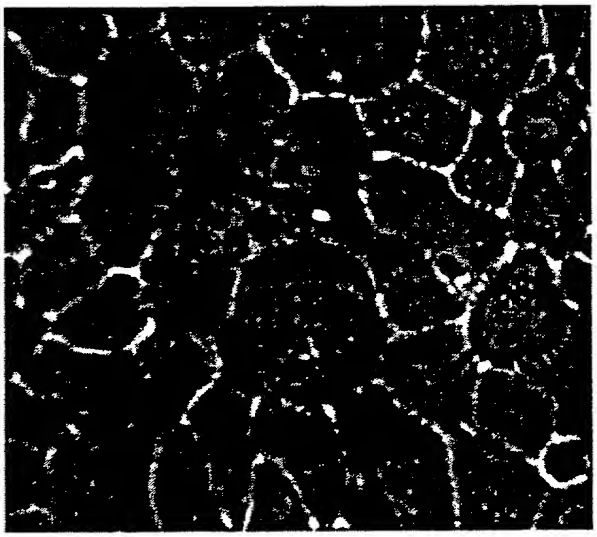


FIG. 1C

1% 4 mM URA Liposomes

FIG. 1D

1% 4 mM URA Liposomes

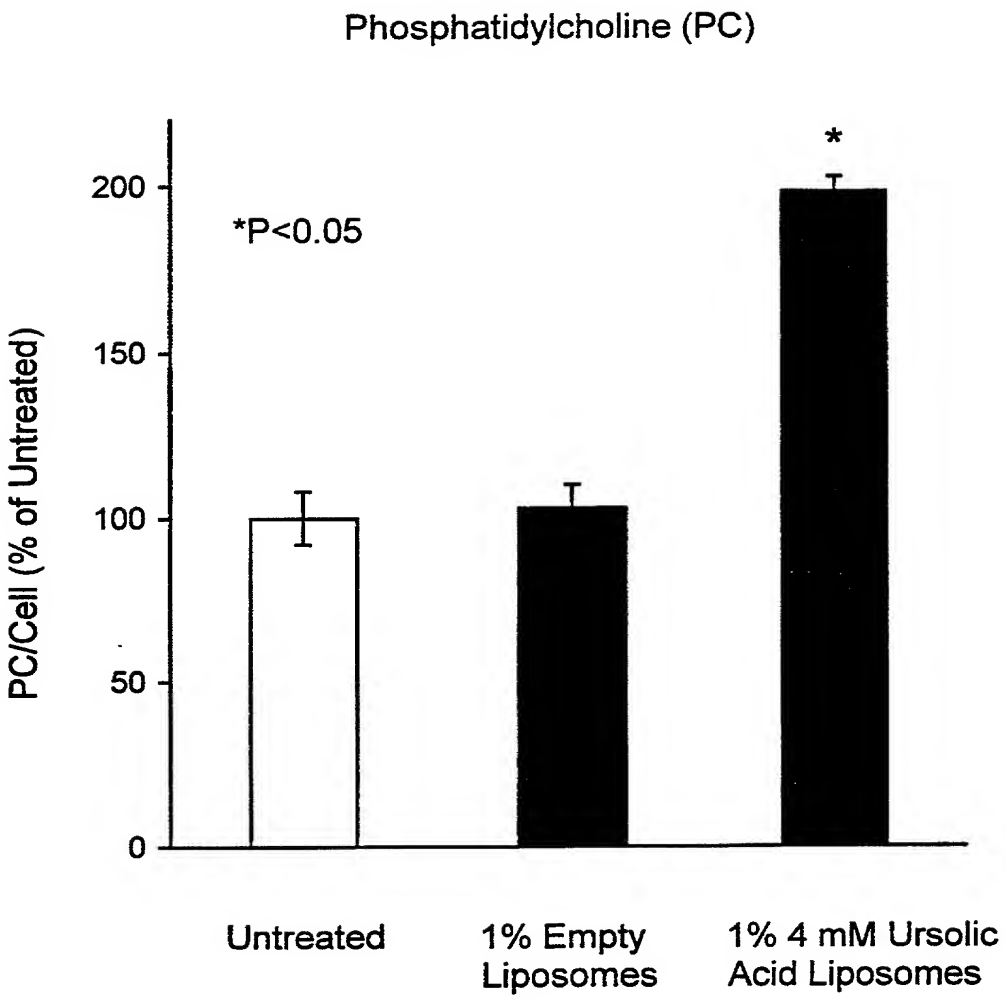

Phosphatidylcholine (PC)
PC/Cell (% of Untreated)
200
150
100
50
0
*P<0.05
*
Untreated
1% Empty Liposomes
1% 4 mM Ursolic Acid Liposomes

FIG. 2

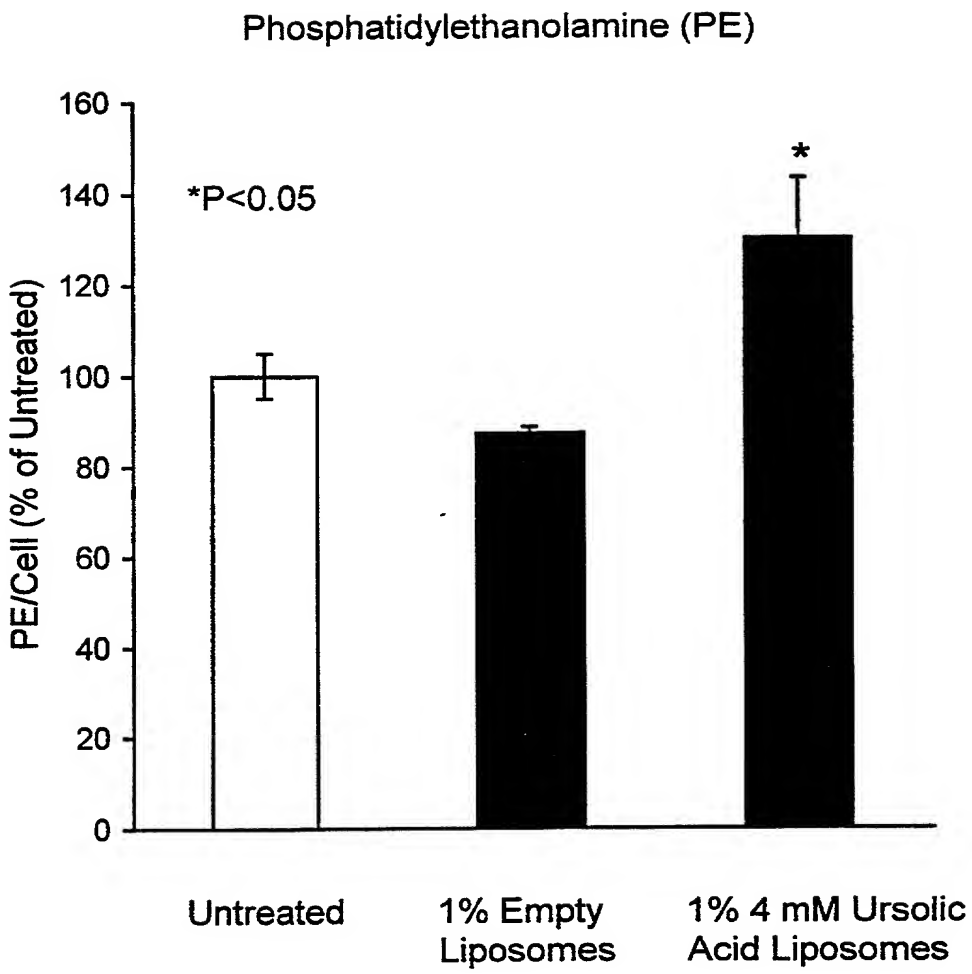

Phosphatidylethanolamine (PE)
160
140
120
100
80
60
40
20
0
PE/Cell (% of Untreated)
*P<0.05
*
Untreated
1% Empty Liposomes
1% 4 mM Ursolic Acid Liposomes

FIG. 3

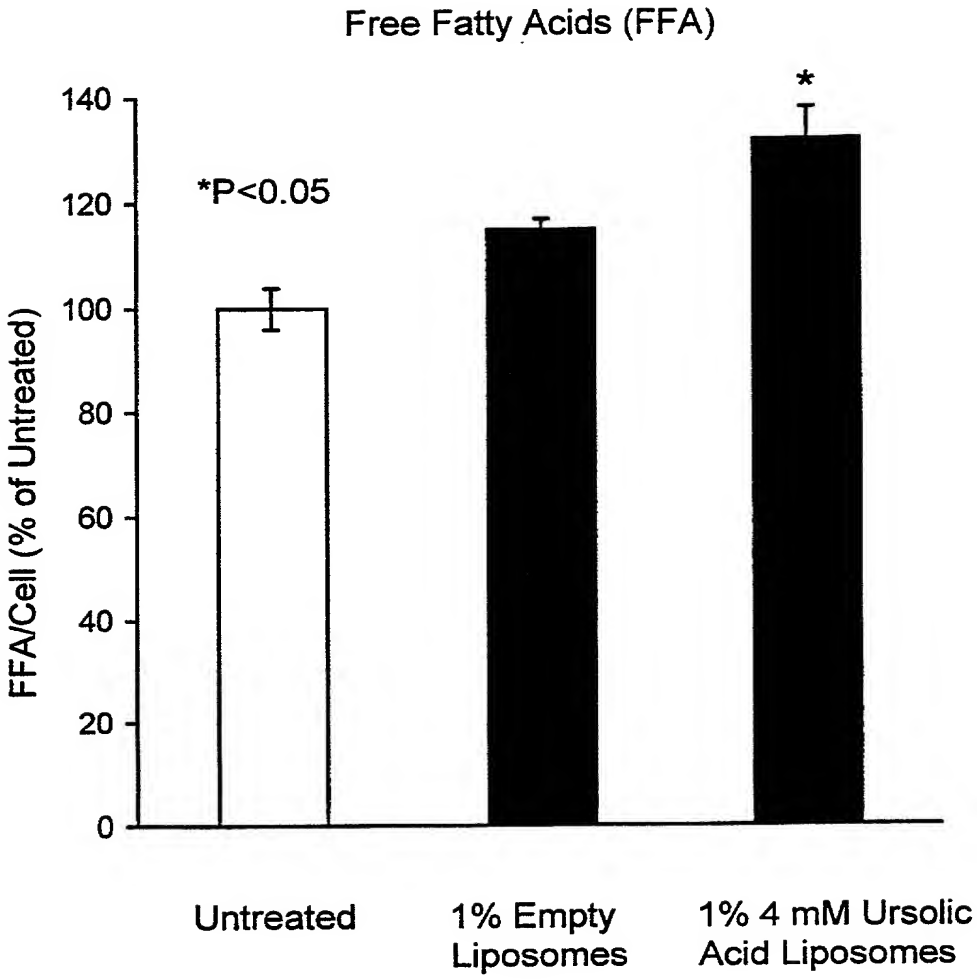

Free Fatty Acids (FFA)
FFA/Cell (% of Untreated)
140
120
100
80
60
40
20
0
*P<0.05
*
Untreated
1% Empty Liposomes
1% 4 mM Ursolic Acid Liposomes

FIG. 4

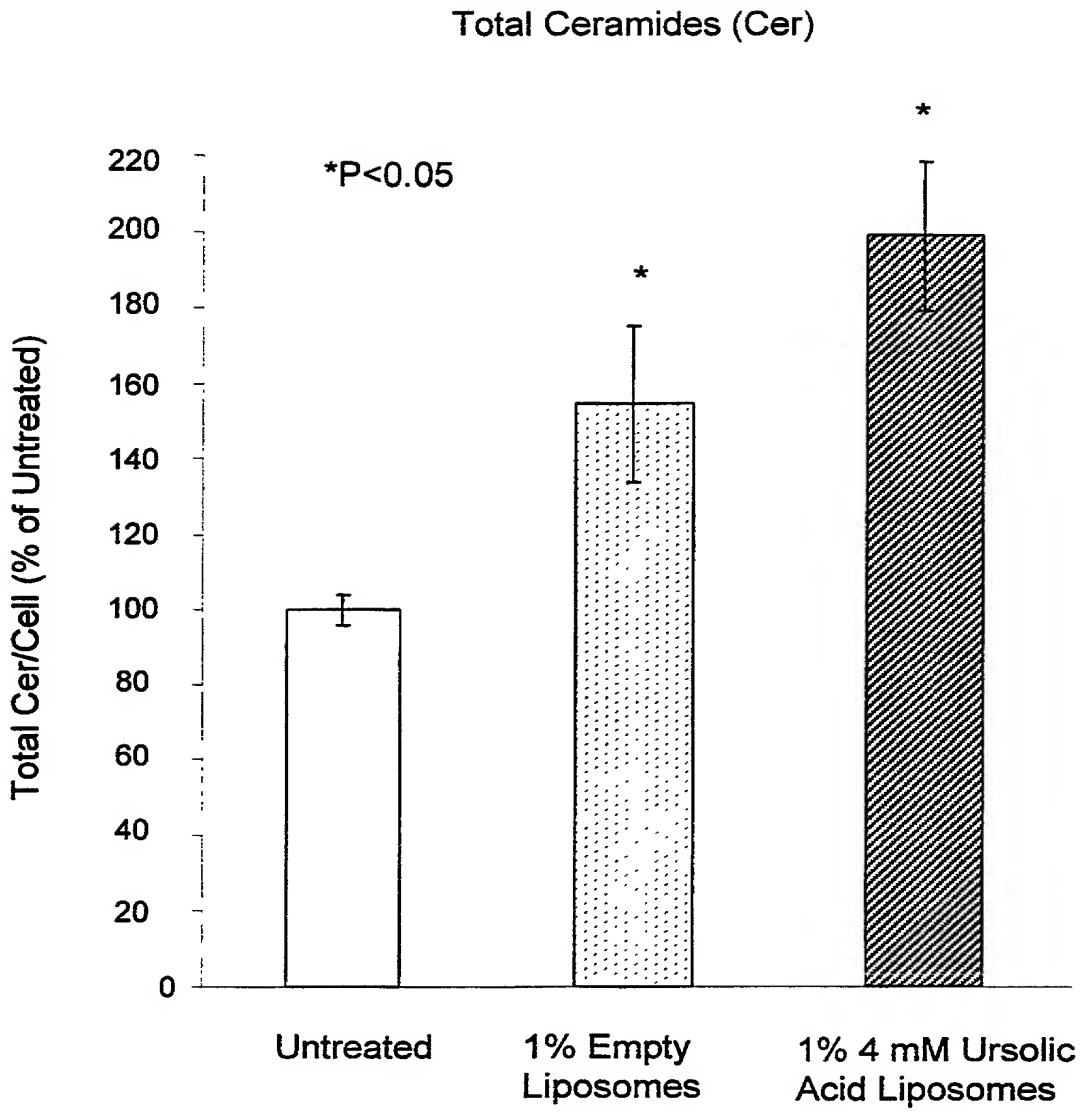

Total Ceramides (Cer)
*P<0.05
Total Cer/Cell (% of Untreated)
220
200
180
160
140
120
100
80
60
40
20
0
*
*
Untreated
1% Empty Liposomes
1% 4 mM Ursolic Acid Liposomes

FIG. 5

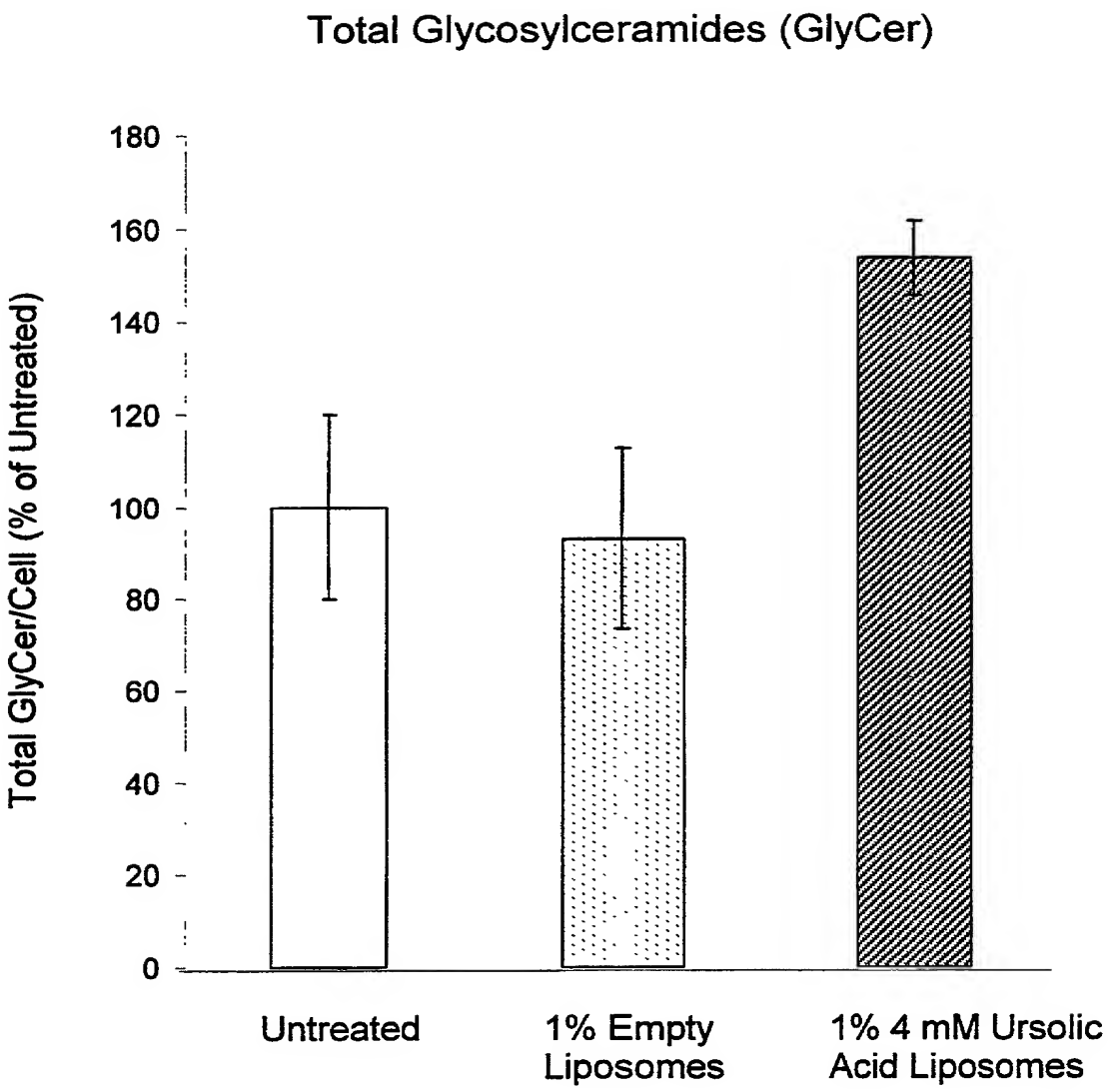

Total Glycosylceramides (GlyCer)
Total GlyCer/Cell (% of Untreated)
180
160
140
120
100
80
60
40
20
0
Untreated
1% Empty Liposomes
1% 4 mM Ursolic Acid Liposomes

FIG. 6

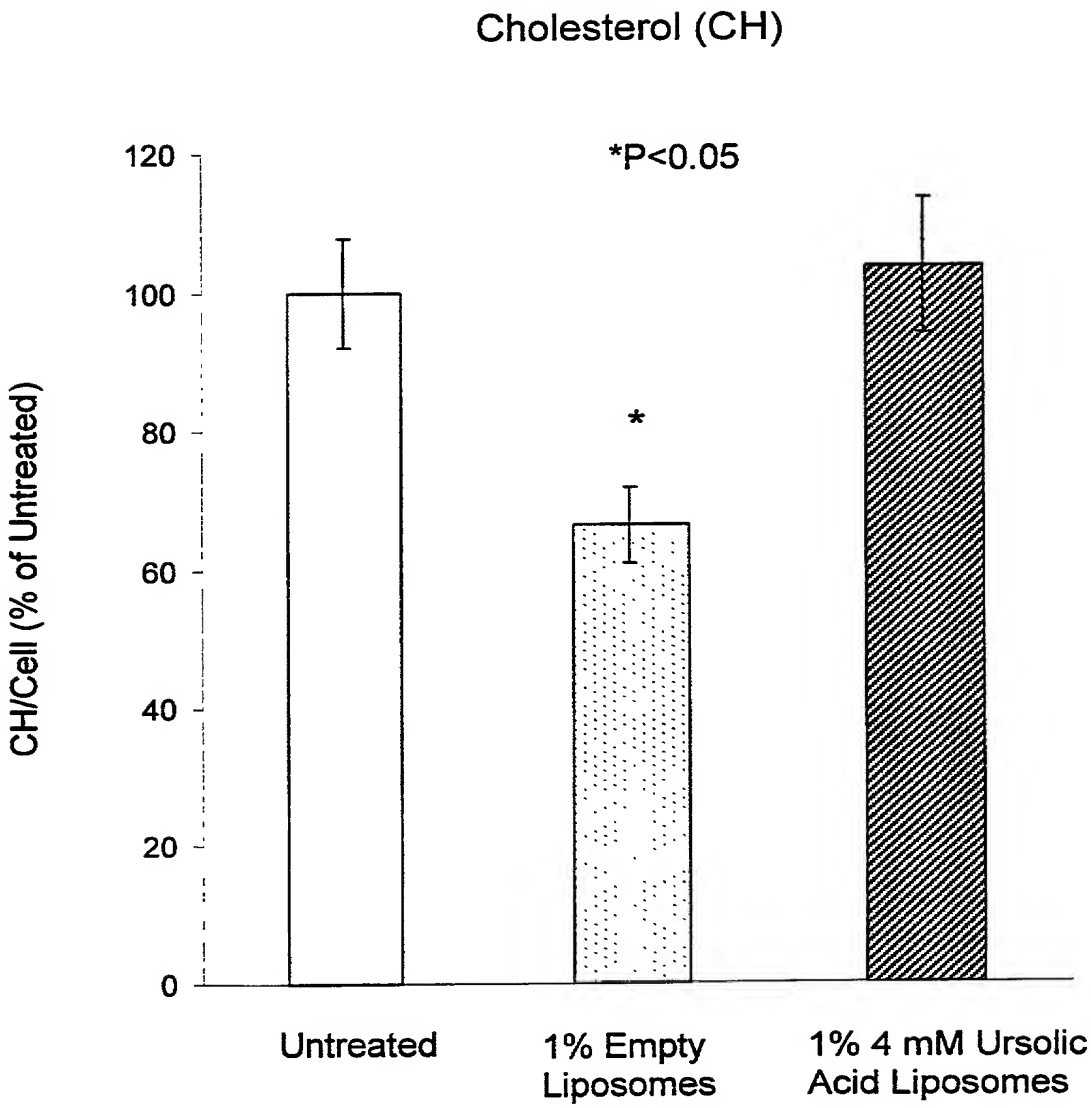

Cholesterol (CH)
*P<0.05
120
100
80
60
40
20
0
CH/Cell (% of Untreated)
*
Untreated
1% Empty Liposomes
1% 4 mM Ursolic Acid Liposomes

FIG. 7

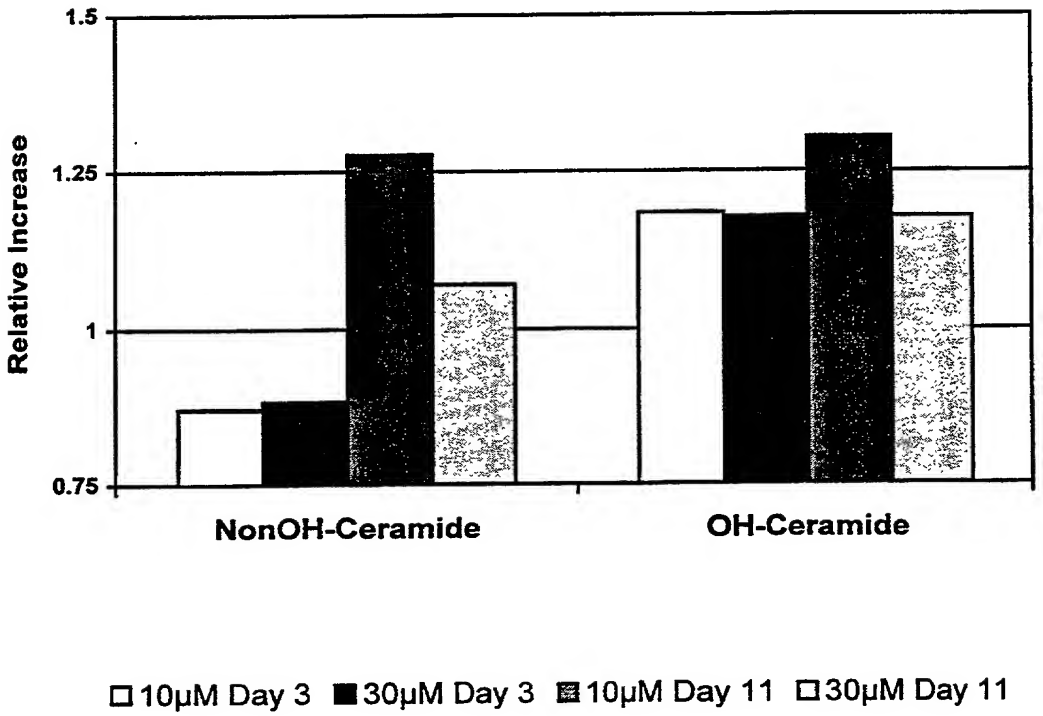

1.5
1.25
1
0.75
Relative Increase
NonOH-Ceramide
OH-Ceramide
10μM Day 3
30μM Day 3
10μM Day 11
30μM Day 11

FIG. 8

Modified PTO/SB/01 (12-97)

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)	Attorney Docket Number	AGI-127
	First Named Inventor	Brown
	COMPLETE IF KNOWN	
	Application Number	
	Filing Date	
	Group Art Unit	
	Examiner Name	

☐ Declaration Submitted with Initial Filing OR ☐ Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16(e)) required)

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

COMPOSITIONS AND METHODS FOR MODIFICATION OF SKIN LIPID CONTENT

(Title of the invention)

the specification of which

☐ is attached hereto
OR
☒ was filed on (MM/DD/YYYY) 09/08/2000 as United States Application Number or PCT International Application Number PCT/US00/24659 and is being amended simultaneously herewith (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached? YES	NO
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)	
60/153,378	09/10/1999	☐ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

DECLARATION - Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (If applicable)

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint the following registered practitioner(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

☐ Customer Number → *Place Customer Number Bar Code Label here*

OR

☒ Registered practitioner(s) name/registration number listed below

Name	Registration Number	Name	Registration Number
Maurice M. Klee	30,399		

☐ Additional registered practitioner(s) named on supplemental Registered Practitioner Information sheet PTO/SB/02C attached hereto.

Direct all correspondence to: ☐ Customer Number or Bar Code Label [ ] OR ☒ Correspondence address below

Name	Maurice M. Klee, Ph.D.						
Address	Attorney at Law						
Address	1951 Burr Street						
City	Fairfield			State	CT	ZIP	06430
Country	US	Telephone	(203) 255-1400			FAX	(203) 254-1101

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

Given Name (first and middle [if any])	Family Name or Surname
David A.	Brown

Inventor's Signature	[signature]					Date	03-05-02
Residence: City	Merrick	State	NY	Country	US	Citizenship	US
Post Office Address	44 Central Parkway						
Post Office Address							
City	Merrick	State	NY	ZIP	11566	Country	US

☒ Additional inventors are being named on the 1 supplemental Additional Inventor(s) sheets PTO/SB/02A attached hereto.

DECLARATION

ADDITIONAL INVENTOR(S)
Supplemental Sheet
Page 1 of 1

2 of

Name of Additional Joint Inventor, if any:							☐ A petition has been filed for this unsigned inventor
Given Name (first and middle (if any))						Family Name or Surname	
Daniel B.						Yarosh	
Inventor's Signature	Daniel B. Yarosh					Date	03/05/02
Residence: City	Merrick	State	NY	Country	US	Citizenship	US
Post Office Address	2915 Shore Drive						
Post Office Address							
City	Merrick	State	NY	ZIP	11566	Country	US

Name of Additional Joint Inventor, if any:							☐ A petition has been filed for this unsigned inventor
Given Name (first and middle (if any))						Family Name or Surname	
Inventor's Signature						Date	
Residence: City		State		Country		Citizenship	
Post Office Address							
Post Office Address							
City		State		ZIP		Country	

Name of Additional Joint Inventor, if any:							☐ A petition has been filed for this unsigned inventor
Given Name (first and middle (if any))						Family Name or Surname	
Inventor's Signature						Date	
Residence: City		State		Country		Citizenship	
Post Office Address							
Post Office Address							
City		State		ZIP		Country	